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One critically important problem in prostate cancer research is to find new approaches to slow down the transition of prostate cancer from an androgen-dependent state to a lethal androgen-refractory state. Intermittent androgen ablation therapy may slow down the development of androgen refractory tumors because intermittent recovery of androgens can induce differentiation of prostatic epithelial cells. However, the advantage of inducing differentiation of prostate cancer cells by intermittent recovery of androgens is compromised by the disadvantage of androgenic induction of prostate cancer cell proliferation. The biologically most active androgen is dihydrotestosterone (DHT), which is converted from testosterone (T) by 5 α -reductase. Our recent studies showed that T is more potent than DHT in enhancing differentiation but weaker in stimulating proliferation, which led to our hypothesis that intermittent androgen suppression (IAS) can be enhanced by finasteride, an inhibitor of T to DHT conversion. We have tested our hypothesis using LNCaP xenograft tumors in nude mice. Our experiments showed that finasteride administration during IAS significantly reduced tumor growth rate and prolonged the life of nude mice bearing LNCaP tumors.

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Introduction:

Conversion of T to DHT is essential for prostate development.

T and DHT are two major biologically active androgens (1). T is synthesized in testis and then transported to target organs, such as the prostate, via blood circulation. T can be converted to DHT in the prostate by 5 α -reductase (2, 3). Both T and DHT bind to the same AR. DHT is more potent than T in activating promoters containing ARE, most likely due to the higher binding affinity of AR to DHT relative to that of T (4-7). The conversion of T to DHT is necessary for normal prostate development because 5 α -reductase inactivation prevents normal prostate development (8, 9). It was thought that the conversion is merely an amplification step for androgen action (10). However, it cannot be ruled out that T and DHT have overlapping yet different biological functions *in vivo*. In fact, our recent studies suggest that T is more potent than DHT in inducing androgen-response genes during the regrowth of the rat ventral prostate (11).

Androgens regulate homeostasis of prostate.

Androgens are required for the structural and functional integrity of the prostate (12). Androgen ablation by castration leads to rapid prostate regression via massive apoptosis (13, 14). On the other hand, androgen replacement stimulates rapid proliferation and differentiation of a regressed prostate until it reaches the normal size (12, 15). Androgen action in a regressed prostate is different from that in the fully-grown prostate because androgens do not stimulate proliferation in a fully-grown prostate (Table 1) (12). During the regrowth of a regressed prostate, androgens induce and then nullify proliferation, establish apoptotic potential while inhibiting apoptosis, and induce and maintain differentiation.

Table 1. The impact of androgen manipulation on the regressed prostate and the normal prostate.

Androgen	Regressed Prostate	Fully-Grown Prostate
+	Proliferation & Differentiation	No Significant Change
-	No Significant Change	Apoptosis & Dedifferentiation

+ represents androgen replacement and – represents androgen ablation or administration of anti-androgens. Differentiation is defined as the expression of prostate-specific markers. Dedifferentiation is defined as loss of prostate-specific marker expression.

Androgen action is intimately associated with prostate cancer pathogenesis.

Androgens are thought to play important roles in prostate cancer pathogenesis (16-18). One of the risk factors for prostate cancer is the presence of the functional testis. Prostate cancer cells are derived from glandular epithelial cells and are initially androgen-dependent. Androgen ablation remains as the standard therapy for metastatic prostate cancer. Unfortunately, androgen ablation therapy is only palliative and eventually patients relapse with androgen-refractory prostate cancer that is currently incurable (18).

Development of androgen-refractory prostate cancer.

The mechanisms of prostate cancer progression from an androgen-dependent state to a lethal androgen-refractory state have been studied extensively. Mutations followed by clonal selection appears to be the mechanism of androgen-independent progression in several prostate cancer models, including the Dunning R3327 rat prostatic adenocarcinoma and LAPC9 human prostate cancer cells (19, 20). Another mechanism for androgen-independent progression involves adaptation. The androgen-independent progression of Shionogi mouse tumor and LNCaP human tumor involve the adaptation (21-24). It is possible that multiple mechanisms are involved in the development of androgen-refractory prostate cancer.

Intermittent androgen ablation therapy.

One urgent challenge in prostate cancer research is to develop new approaches to inhibit or to slowdown the development of androgen-refractory prostate cancer. Intermittent androgen ablation therapy was developed, attempting to delay the emergence of androgen-refractory prostate tumors relative to the continuous androgen ablation therapy. The rationale is that intermittent recovery of androgens can promote prostate cancer cell differentiation and enhance their dependence on androgens (24, 25). However, androgens are also proliferative to prostate cancer cells, which is undesirable in the therapy. The goal of our proposal is to increase the efficacy of intermittent androgen suppression by enhancing the differentiation effects while inhibiting the proliferative effects via finasteride administration.

Finasteride enhances the expression of many androgen-response genes during T-stimulated regrowth of the regressed prostate.

One interesting question in androgen action is whether or not the expression of androgen-response genes is differentially regulated by T and DHT. Finasteride, a 5 α -reductase inhibitor, had little or no effect on the expression of the surveyed androgen-response genes in testis-intact rats (11). However, the induction of half of the surveyed androgen-response genes, including prostatein C3, adrenomedullin and calreticulin, are further enhanced by finasteride during T-stimulated regrowth of a regressed rat ventral prostate (Fig. 3) (11). This unexpected observation suggests that T is more potent than DHT in inducing androgen-response genes in prostate regrowth.

Since finasteride only enhances androgen-response gene expression in a regressed prostate but not in a fully-grown prostate, finasteride is expected to enhance the expression of androgen-response genes in prostate tumor regrowth induced by intermittent recovery of androgens but not in prostate tumors untreated with androgen ablation therapy.

Body:

Task 1: Determine quantitatively the relative potency of T versus DHT in the induction of androgen-response genes during the prostate regrowth (Month 1-36).

- a. Animal manipulation and collecting prostatic tissue and serum samples.
- b. Measurement of serum and intraprostatic T and DHT.
- c. Measurement of DNA contents in the rat ventral prostate in the presence and absence of finasteride.
- d. Northern and Western blot analysis of androgen-response gene expressions in the rat ventral prostate in the presence or absence of finasteride.

We did not focus on Task 1 last year because Task 2 is the key to the project and we have already presented some data in our first annual report. Without the success of Task 2, the whole project may have very little clinical relevance. Also, the success of Task 2 would facilitate the accomplishment of Task 1 and Task 3. During the 3rd year of the funding period, we continued our focus on Task 2.

Task 2: Test the effect of finasteride on intermittent androgen ablation therapy of xenograft androgen-sensitive prostate tumors in nude mice (Months 1-36).

- a. Establish LNCaP androgen-sensitive tumor models in nude mice.
- b. Determine the impact of finasteride on intermittent androgen ablation in Shionogi model by collecting tumor specimens and serum samples for analysis.
- c. Determine the impact of finasteride on the time required to establish androgen-independent PSA expression in LNCaP tumor model undergoing intermittent androgen suppression.
- d. Determine whether finasteride administration during the "off-cycle" of intermittent androgen ablation therapy will prolong the survival of nude mice with subcutaneous LNCaP xenograft tumors (**New task**).

One cycle of intermittent androgen ablation. At the end of one cycle of intermittent androgen ablation (orchiectomy followed by pellet administration, Figure 1), the mean percent change in tumor volume (\pm SEM) during the 'off-cycle' was similar in the CAA, F, and T groups ($114 \pm 22\%$, $91 \pm 46\%$, and $128 \pm 18\%$, respectively (Figure 2a). Mice treated with T+F during the 'off-cycle' experienced less tumor growth ($23 \pm 13\%$, $p=0.002$). Serum PSA did not significantly differ between the four groups (data not shown).

We then stratified the results by the percentage of mice that experienced no change or a decrease in tumor growth at the end of the initial cycle of therapy (Figure 2b). No significant differences

were seen between the CAA, F, and T groups (12%, 0%, and 10%, respectively). An increased percentage of mice treated with T+F during the 'off-cycle' experienced no change or a decrease in tumor volume (41%).

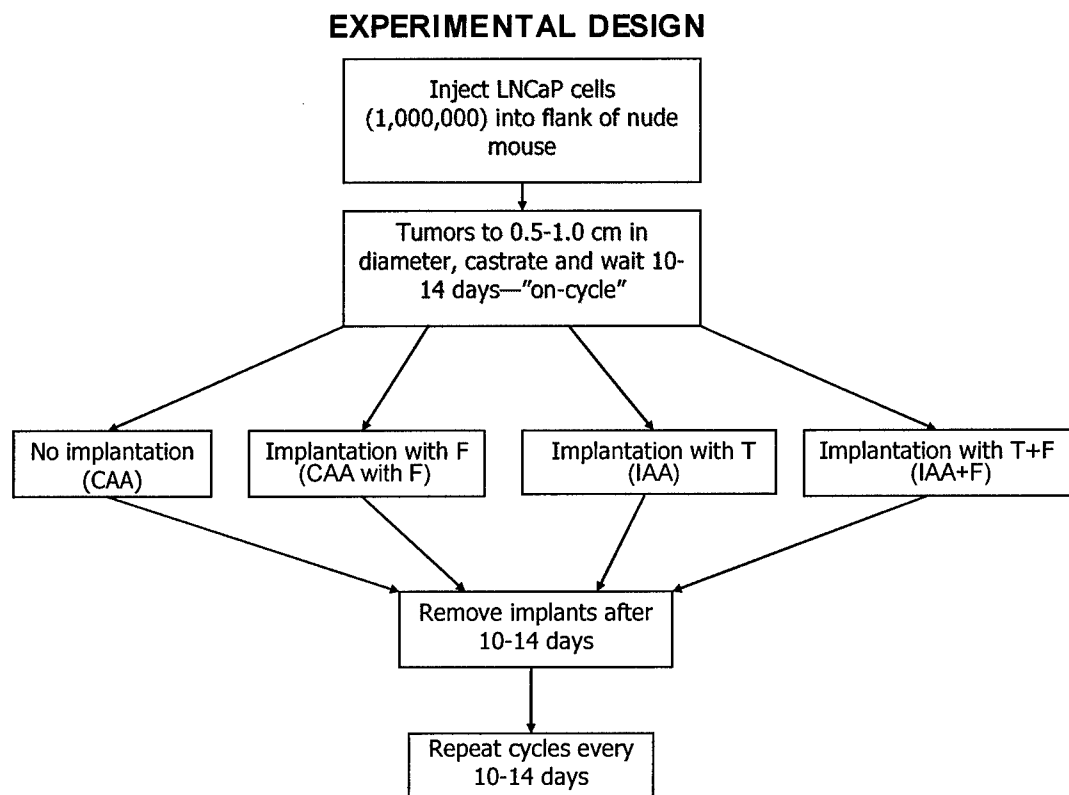


Figure 1. Treatment strategy of LNCaP xenograft tumor in nude mice. After the tumor establishment, animals are castrated for 10-14 days, which is considered as the "on-cycle" of androgen ablation during IAA. The animals are then implanted with no pellet, testosterone (T) pellet, finasteride (F) pellet, or both pellets for 14 day or longer. Testosterone implantation mimics intermittent recovery of testicular function during the "off-cycle" of IAA. Controls are continuous androgen ablation (CAA) in the absence or presence of finasteride (F).

Outcomes based on initial tumor volume and treatment. To evaluate if the differences seen between treatment groups were related to initial tumor size, we divided the mice into three groups based on volume of tumor at the time of treatment randomization ($<0.33 \text{ cm}^3$, $0.33\text{-}1.0 \text{ cm}^3$, and $>1.0 \text{ cm}^3$). In all three groups, the mice treated with T+F experienced significantly less tumor growth during the first cycle compared to mice treated by CAA or T alone ($<0.33 \text{ cm}^3$: 62% vs 129% vs 172%, $p=0.038$; $0.33\text{-}1.0 \text{ cm}^3$: 35% vs 150% vs 247%, $p=0.0022$; and $>1.0 \text{ cm}^3$: 47% vs 130% vs 134%, $p=0.027$; Figure 2c). The limited number of mice in the F group prevented their inclusion in the analysis.

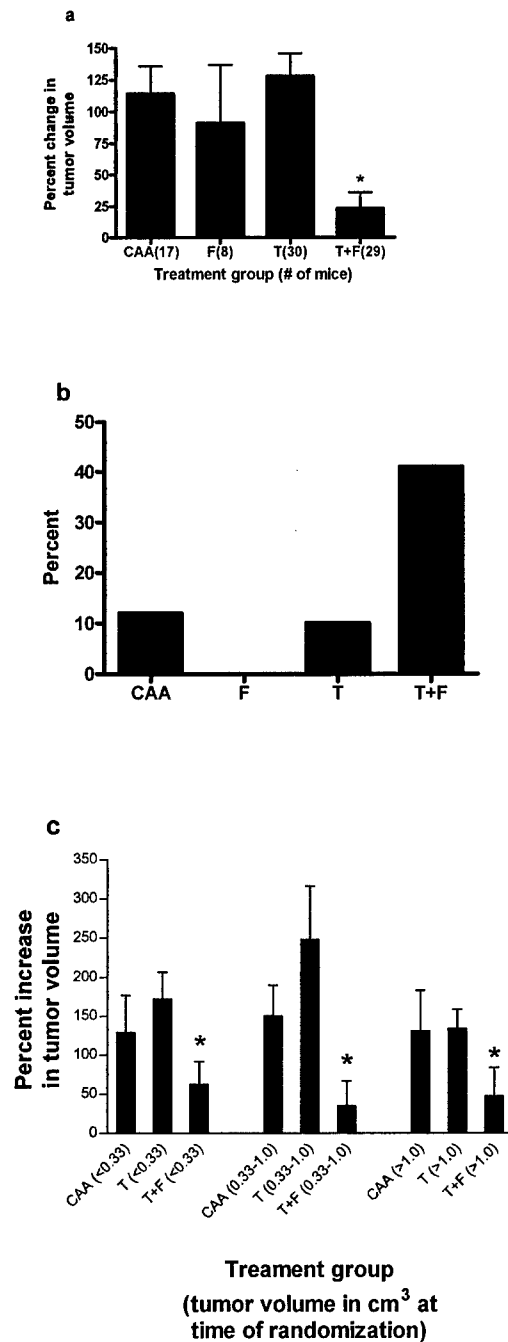


Figure 2: a) Mean percent change in LNCaP tumor volume (±SEM) during the first 'off-cycle' of intermittent androgen ablation (p=0.0002), b) percentage of mice with no increase in tumor volume at the end of one cycle of intermittent androgen ablation, c) percent change in tumor volume (±SEM) stratified by size of tumor at time of treatment randomization (CAA vs T vs T+F). p-values for <0.33 cm³, 0.33-1.0 cm³, and >1.0 cm³ are 0.038, 0.0022, and 0.027, respectively. * indicates statistically significant

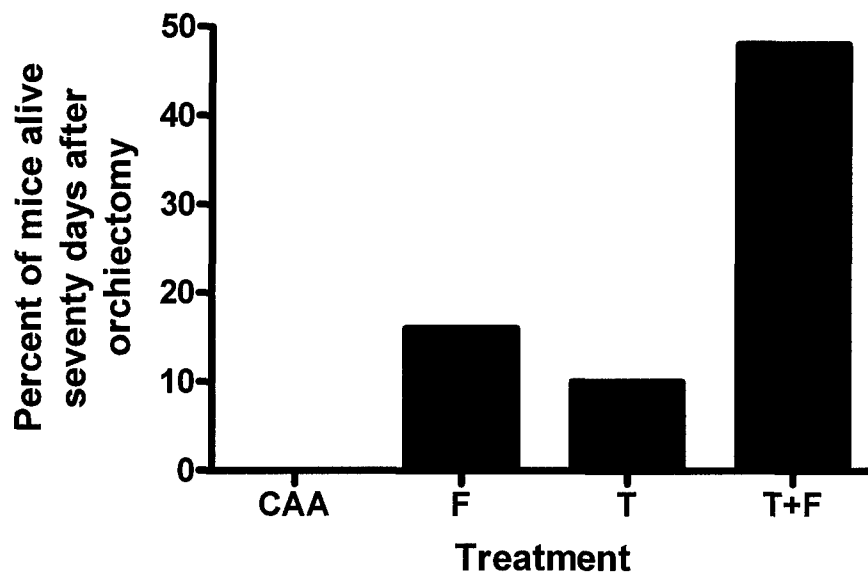
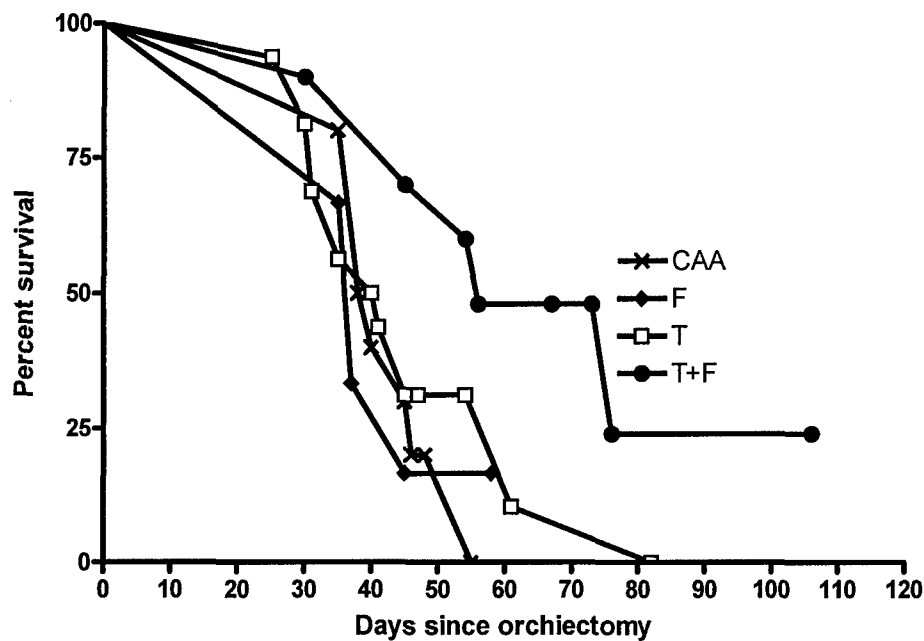


Figure 3: a) Kaplan-Meier survival curve of intermittent androgen ablation with 'off-cycle' treatments (CAA-continuous androgen ablation, F-finasteride, T-testosterone, and T+F-testosterone plus finasteride). Euthanasia was performed if tumor diameter > 2.0 cm, tumor ulceration, or tumor-related morbidity. Log-rank test for trend, $p=0.048$, b) percent survival seventy days following orchiectomy.

Survival analysis of intermittent androgen ablation. To evaluate if changes in tumor volume correlated with a survival benefit, we next performed a survival analysis. Mice randomized to the T+F treatment group had the best survival, defined as death or time to euthanasia, seen at all time points (Figure 3a, log rank p-value=0.048). Both median survival and survival seventy days following treatment also favored the T+F group (Figure 3b). Compared to the other treatment groups, T+F mice were 3-5 times more likely to be alive seventy days following treatment.

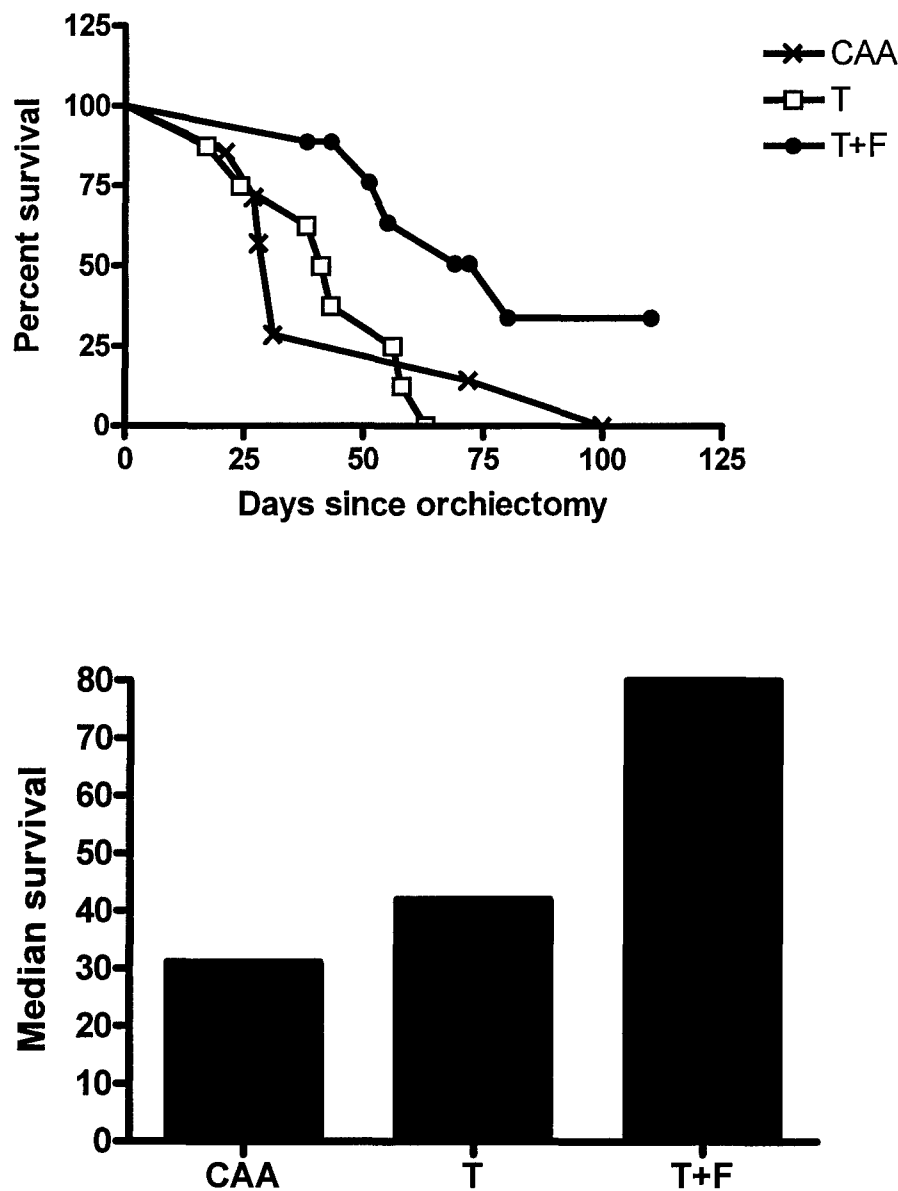


Figure 4: a) Kaplan-Meier survival curve of one-time continuous pellet administration following 14 days of castration (CAA-continuous androgen ablation, T-testosterone, and T+F-testosterone plus finasteride), $p=0.034$, b) median survival.

Survival analysis of one-time pellet implantation. Since the duration of human ‘off-cycle’ intermittent androgen ablation can vary widely, we next evaluated survival following one-time continuous pellet implantation, in essence an extended ‘off-cycle’. Mice treated with T+F had the best survival ($p=0.034$, Figure 4a) and longest median survival (T+F: 80 days, T: 42 days, CAA: 31 days, Figure 4b).

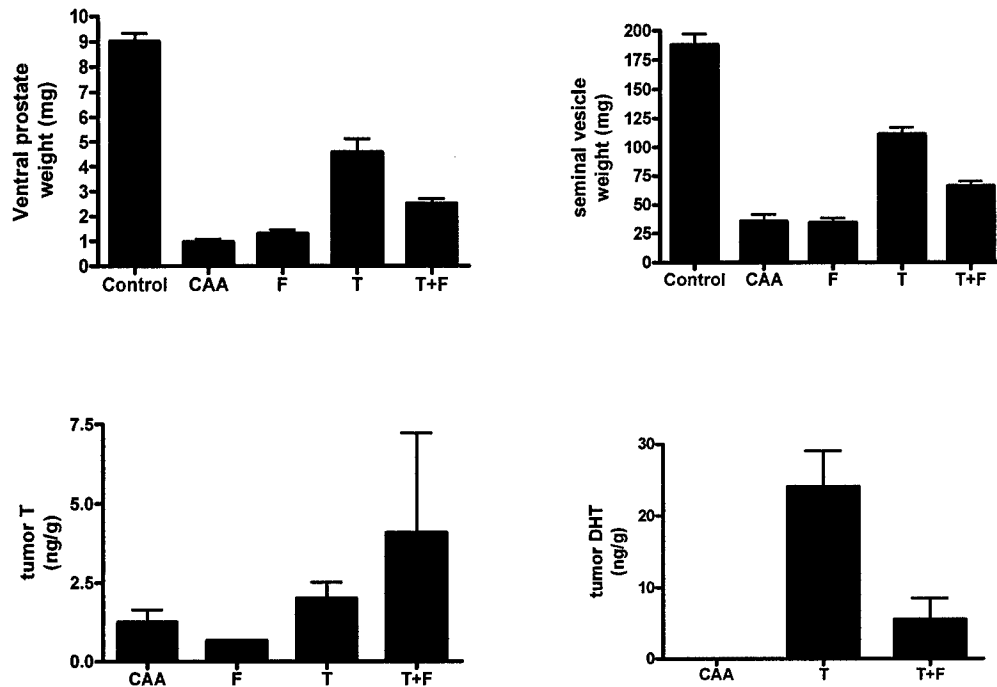


Figure 5: a) ventral prostate weights (\pm SEM) following fourteen days of pellet implantation; T versus T+F, $p=0.005$, b) seminal vesicle weights (\pm SEM) following fourteen days of pellet implantation; T versus T+F, $p<0.001$, c) tissue (ng/g) concentrations of testosterone (\pm SEM), $p=0.37$; d) tumor (ng/g) concentrations (\pm SEM) of dihydrotestosterone, $p=0.008$.

Hormonal evaluation. The adequacy of the testosterone and finasteride pellets was tested by measuring ventral prostate and seminal vesicle weights for each treatment group (Figure 5). Testosterone-treated mice experienced the largest mean ventral prostatic and seminal vesicle growth (Figures 5a-b, p -values for both <0.0001) but only returned to 50% of non-castrated mouse prostate weight. Compared to testosterone-treated mice, the implantation of finasteride in addition to testosterone resulted in a 46% and 41% decrease, respectively, in mean ventral prostate ($p=0.005$) and seminal vesicle weights (Figures 5a-b, $p=0.005$ and $p<0.001$, respectively). While mean tumor T concentrations did not statistically differ between the T-treated and T+F-treated mice (Figure 5c), mean tumor DHT concentrations were lower in the T+F-treated mice with a trend towards significance (Figure 5d, p -value=0.064). Since

testosterone can be converted to estradiol, we next evaluated if elevated serum or tumor estradiol levels may account for the tumor growth inhibition seen in the T+F-treated mice. There were no differences in the serum estradiol levels between the treatment groups, with all levels below 75 pg/ml (data not shown). Estradiol levels were undetectable in the tumor tissue.

No significant difference in serum PSA levels was detected among the four groups of nude mice. Serum was obtained by retro-orbital venipuncture. Serum PSA levels were determined by a commercial kit (IMx PSA, Abbott Laboratories, Abbott Park, IL). Serum PSA did not significantly differ between the four groups (data not shown). This finding indicates that LNCaP xenograft tumors in our experiment are already on the way to become independent of androgens, which is consistent with their growth in castrated hosts (Figure 2). This finding also means that it will virtually be impossible for us to use androgen-independent PSA expression as an endpoint in our proposed studies (Task 2c).

Task 3: Determine the effect of finasteride on the expression of androgen-response genes in LNCaP tumors during intermittent androgen ablation therapy (Month 24-36).

- a. Collect LNCaP tumor specimens and serum samples from nude mice.
- b. Determine the expression of androgen-response genes, adrenomedullin, calreticulin and PSA, in LNCaP tumors.
- c. Analysis of the collected data and prepare the final report for the proposal.

Animal studies are time consuming and labor intensive. Also, LNCaP xenograft tumor take rate and growth rate have high variation. These difficulties have slowed down our progresses. We are in the process of performing this Task 3. LNCaP xenograft tumors have being established in nude mice. We expect to complete this study in next few months.

Key Research Accomplishments:

Our proposed research requires the ability to deliver appropriate doses of finasteride and T over a prolonged period in nude mice. In the 1st year of the funding period, we have encountered difficulties because the commercially available slow releasing pellets did not work in our system. After multiple tests, we resolved these critical technical problems, which allowed us, in the 2nd year of the funding period, to demonstrate that finasteride administration

- significantly enhances the efficacy of intermittent androgen ablation therapy in LNCaP xenograft tumor model.

1. **We demonstrated that finasteride given during the 'off-cycle' of intermittent androgen ablation significantly limits tumor growth in the LNCaP xenograft model.** The use of IAS plus finasteride resulted in decreased tumor growth compared to standard continuous androgen ablation. We are not aware of any previous report showing that parental LNCaP tumor size is reduced by hormonal manipulation other than castration.

2. **Finasteride maintenance during the "off-cycle" of intermittent androgen ablation prolonged the survival of nude mice bearing the LNCaP xenograft tumors.** Our finding provide a strong basis for the further studies on the potential survival benefits of finasteride "off-cycle" maintenance for prostate cancer patients undergoing IAS treatment.

Reportable Outcomes:

1. We have a manuscript under revision in the PROSTATE (See attached document).

Conclusions:

Our studies with androgen-sensitive LNCaP human prostate tumor xenografts in nude mice showed significant tumor growth retardation by finasteride plus IAS in the first cycle. Our finding showed that finasteride plus IAS prolongs the life of nude mice bearing LNCaP tumors. Also, this finding suggests that finasteride administration should enhance the efficacy of IAS on patients with prostate cancer.

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Appendices:

1. Eggener SE, Stern, JA, Oram S, Cai, X, Roehl K, Wang Z: Enhancement of intermittent androgen ablation by "off-cycle" maintenance with finasteride in LNCaP prostate cancer xenograft model, In revision in *PROSTATE* (Attached).

**ENHANCEMENT OF INTERMITTENT ANDROGEN ABLATION BY 'OFF-CYCLE'
MAINTENANCE WITH FINASTERIDE IN LNCaP PROSTATE CANCER
XENOGRAFT MODEL**

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**Key Words: finasteride, prostate cancer, LNCaP, testosterone, intermittent androgen
ablation**

Running Title: Intermittent Androgen Ablation with Finasteride

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ABSTRACT

Background: Intermittent androgen ablation (IAA) was developed with the intention of delaying progression of prostate cancer to androgen-independence and improving quality of life. Our previous studies suggest that relative to dihydrotestosterone (DHT), testosterone (T) is weak in inducing proliferation and more potent in inducing differentiation. We hypothesize that administration of finasteride (F), a type-II 5- α -reductase inhibitor, during the IAA 'off-cycle' would enhance the efficacy.

Methods: After LNCaP tumor establishment, nude mice were castrated and randomized to continuous androgen ablation (CAA), continuous androgen ablation plus finasteride (F), intermittent androgen ablation (IAA), or intermittent androgen ablation plus finasteride (IAA+F).

Results: After one cycle of therapy, mice treated with IAA+F had significantly less tumor growth than the other treatment groups ($p=0.002$). Mice treated with IAA+F had the best survival ($p=0.048$) and were 3-5 times more likely to be alive seventy days following treatment initiation.

Conclusions: Intermittent androgen ablation with finasteride provides the most favorable tumor growth kinetics and survival compared to both continuous and standard intermittent androgen ablation.

INTRODUCTION

Prostate cancer remains the second leading cause of non-skin cancer related death among American men. Since its growth is hormone-dependent, androgen ablation is a common and increasingly utilized treatment(1). While initially effective at inducing tumor regression in most men, it is palliative and not curative, as all tumors will eventually become refractory to hormonal therapy. In addition to the treatment limitations, reduced androgen levels lead to weight gain, dryness of skin, hot flashes, diminished libido, impotence, cognitive dysfunction, and muscle loss(2). In an attempt to minimize the duration and severity of these side effects, intermittent androgen ablation (IAA) was developed(3). Medical castration is followed by a period of androgen recovery, during which serum testosterone slowly returns to normal levels. Upon tumor progression (typically a pre-determined PSA level), androgen ablation is reinstituted. These cycles of “on” (androgen ablation) and “off” (androgen recovery) treatment may be repeated until the tumor no longer responds favorably to hormonal manipulation. During intervals of androgen recovery, quality-of-life parameters are more favorable compared to the periods of androgen ablation. In addition to the symptomatic benefits, intermediate-term clinical studies suggest IAA does not compromise cancer control when compared to CAA(4). In a mouse xenograft model, the time to androgen-independence may even be delayed compared to CAA(3,4).

Testosterone is converted to dihydrotestosterone (DHT) by 5- α -reductase. We have previously identified a cohort of genes expressed during the regrowth of castrated rat prostate (5,6). A subset of these genes exhibit variable expression profiles when exposed to different levels of testosterone or dihydrotestosterone (DHT). For example, following castration testosterone is a

more potent inducer of many androgen-responsive genes compared to DHT(7). Some of these genes encode growth-inhibiting proteins (e.g. U19, ALP1, adrenomedullin) (8-10). A testosterone-rich, DHT-poor environment induces expression of growth-inhibiting proteins only during regrowth of a previously androgen-ablated prostate but is not observed in the intact, untreated prostate (7). While the Prostate Cancer Prevention Trial (PCPT) showed a 5- α -reductase inhibitor (finasteride) in hormonally-intact men can decrease the incidence of prostate cancer by 25%(11), it does not have beneficial effects in treating clinical prostate cancer.

Therefore, the prostatic regrowth phase of IAA appeared to be an appropriate clinical model to test if a testosterone-rich, DHT-poor environment would result in more favorable tumor growth kinetics. We hypothesized that use of finasteride to maximize testosterone and limit DHT during the androgen recovery stage of IAA would result in slower prostatic regrowth, reduced proliferation, and improved cancer control. The LNCaP tumor cell line was selected as our tumor model because it is an androgen-sensitive, PSA-secreting cell line derived from the lymph node of a man with metastatic prostate cancer. The type II 5- α -reductase is the predominant isoenzyme in human prostate and also present in the human-derived prostate cancer cell line, LNCaP(12).

MATERIALS AND METHODS

Cell culture: LNCaP cells were obtained from American Type Culture Collection (ATCC) and grown in sterilized medium containing RPMI 1640, L-glutamine, penicillin/streptomycin, and fetal bovine serum (FBS) under an atmosphere of 5% CO₂. Cells underwent 4-10 passages prior to mouse inoculation. Experiments were performed with two separate batches of LNCaP cells.

The tumor volume and survival results detailed below were reproducible in both sets of LNCaP cells studied.

Animal experiments: All animal experiments were approved by the Northwestern University Animal Care Use Committee. The design of animal experiments is diagramed in Figure 1. Approximately 1×10^6 LNCaP cells were inoculated subcutaneously with 0.25 ml of Matrigel (Becton Dickinson, Bedford, MA) in the flank region of 6-8 week old athymic male mice. Of the injected mice developing tumors (~75%), they typically exhibited visible tumor growth 8-12 weeks following inoculation. Tumors were allowed to grow until they reached 5-10 mm in diameter. All mice were then castrated via a trans-scrotal approach under tribromoethanol anesthesia and considered 'on-cycle'. Ten to fourteen days following castration, the mice were assigned to one of four groups and considered 'off-cycle': 1) continuous androgen ablation (CAA; no implants), 2) control group (finasteride [F] pellet implants), 3) intermittent androgen ablation (IAA; testosterone [T] pellet implants), and 4) intermittent androgen ablation with finasteride (IAA+F; testosterone [T] and finasteride [F] implants). Silicone pellets of either testosterone or finasteride were implanted subcutaneously in the flank contralateral to the tumor. The mice were distributed so mean tumor volume at time of randomization was equivalent among the four groups. The pellets were extracted after an implantation period of ten to fourteen days and this constituted the end of one full cycle. Cycles were repeated until mouse death, tumor overgrowth (> 2 cm in diameter), tumor ulceration, or severe tumor-related morbidity required euthanasia. Tumor volume was calculated as $(\text{length} \times \text{width}^2)/2(13)$. Serum was obtained by retro-orbital venipuncture. Tumor volume and serum PSA were measured at each

intervention. Tumor was flash-frozen with liquid nitrogen at the time of euthanasia and stored at -20°C for later determination of hormonal concentrations.

Pellet construction: Silicone tubing with a 1.58mm internal diameter and a 3.18mm outer diameter (Catalog #60-411-47; Helix Medical, Carpinteria, CA) was cut to 8mm in length. A wood stick of 1.58mm diameter was inserted 3mm into one end of the silicone tubing. From the other end of tubing, 2mm (approximately 7.6 mg) of testosterone (Sigma Chemical, St Louis, MO) was tightly packed. The remaining open end of silicone tubing was filled with another wood stick. The portions of wood extending beyond the silicone tubing were cut with a razor blade. Both ends of the pellet were sealed with silicone adhesive (Product code #00698, DAP®) and allowed to air dry overnight. Finasteride pellets were made in a similar fashion with a few exceptions. Finasteride was a generous gift of Merck (Rahway, NJ). Silicone tubing had a 1.47mm internal diameter and 1.96mm outer diameter (Catalog #60-411-45; Helix Medical, Carpinteria, CA). Total tube length was 2cm long with 12mm (approximately 15mg) of finasteride powder flanked by wood sticks filling 4mm on each side. Following the overnight adhesive drying, both testosterone and finasteride pellets were then sterilized with 70% ethanol for 10 minutes and stored in a light-free environment. Adequacy of the pellets was evaluated by their effect on the weights of ventral prostate and seminal vesicles following subcutaneous flank implantation into castrated athymic mice for 14 days.

Study endpoints: Study endpoints were tumor volume, serum PSA, and mouse survival. Euthanasia was performed if the tumor diameter exceeded 2 cm, ulcerated or caused severe tumor-related morbidity. Serum PSA levels were determined by a commercial kit (IMx PSA,

Abbott Laboratories, Abbott Park, IL). Radioimmunoassay kits (Diagnostic Systems Laboratories, Inc., Webster, TX) were used to measure serum and tumor T, DHT, and estradiol (E2).

Statistical analysis: GraphPad Prism 4.0 was used for all statistical analyses and graphical composition. Tumor volume, prostate and seminal vesicle weight, and hormonal levels were compared using non-parametric one-way ANOVA. Survival analysis was evaluated using Kaplan-Meier curves and log rank tests. A p-value <0.05 was considered statistically significant.

RESULTS

One cycle of intermittent androgen ablation. At the end of one cycle of intermittent androgen ablation (orchiectomy followed by pellet administration, Figure 1), the mean percent change in tumor volume (\pm SEM) during the 'off-cycle' was similar in the CAA, F, and T groups ($114 \pm 22\%$, $91 \pm 46\%$, and $128 \pm 18\%$, respectively (Figure 2a). Mice treated with T+F during the 'off-cycle' experienced less tumor growth ($23 \pm 13\%$, $p=0.002$). Serum PSA did not significantly differ between the four groups (data not shown).

We then stratified the results by the percentage of mice that experienced no change or a decrease in tumor growth at the end of the initial cycle of therapy (Figure 2b). No significant differences were seen between the CAA, F, and T groups (12%, 0%, and 10%, respectively). An increased percentage of mice treated with T+F during the 'off-cycle' experienced no change or a decrease in tumor volume (41%).

Outcomes based on initial tumor volume and treatment. To evaluate if the differences seen between treatment groups were related to initial tumor size, we divided the mice into three groups based on volume of tumor at the time of treatment randomization ($<0.33 \text{ cm}^3$, $0.33\text{-}1.0 \text{ cm}^3$, and $>1.0 \text{ cm}^3$). In all three groups, the mice treated with T+F experienced significantly less tumor growth during the first cycle compared to mice treated by CAA or T alone ($<0.33 \text{ cm}^3$: 62% vs 129% vs 172%, $p=0.038$; $0.33\text{-}1.0 \text{ cm}^3$: 35% vs 150% vs 247%, $p=0.0022$; and $>1.0 \text{ cm}^3$: 47% vs 130% vs 134%, $p=0.027$; Figure 2c). The limited number of mice in the F group prevented their inclusion in the analysis.

Survival analysis of intermittent androgen ablation. To evaluate if changes in tumor volume correlated with a survival benefit, we next performed a survival analysis. Mice randomized to the T+F treatment group had the best survival, defined as death or time to euthanasia, seen at all time points (Figure 3a, log rank p-value=0.048). Both median survival and survival seventy days following treatment also favored the T+F group (Figure 3b). Compared to the other treatment groups, T+F mice were 3-5 times more likely to be alive seventy days following treatment.

Survival analysis of one-time pellet implantation. Since the duration of human 'off-cycle' intermittent androgen ablation can vary widely, we next evaluated survival following one-time continuous pellet implantation, in essence an extended 'off-cycle'. Mice treated with T+F had the best survival ($p=0.034$, Figure 4a) and longest median survival (T+F: 80 days, T: 42 days, CAA: 31 days, Figure 4b).

Hormonal evaluation. The adequacy of the testosterone and finasteride pellets was tested by measuring ventral prostate and seminal vesicle weights for each treatment group (Figure 5). Testosterone-treated mice experienced the largest mean ventral prostatic and seminal vesicle growth (Figures 5a-b, p-values for both <0.0001) but only returned to 50% of non-castrated mouse prostate weight. Compared to testosterone-treated mice, the implantation of finasteride in addition to testosterone resulted in a 46% and 41% decrease, respectively, in mean ventral prostate ($p=0.005$) and seminal vesicle weights (Figures 5a-b, $p=0.005$ and $p<0.001$, respectively). While mean tumor T concentrations did not statistically differ between the T-treated and T+F-treated mice (Figure 5c), mean tumor DHT concentrations were lower in the T+F-treated mice with a trend towards significance (Figure 5d, p-value=0.064). Since

testosterone can be converted to estradiol, we next evaluated if elevated serum or tumor estradiol levels may account for the tumor growth inhibition seen in the T+F-treated mice. There were no differences in the serum estradiol levels between the treatment groups, with all levels below 75 pg/ml (data not shown). Estradiol levels were undetectable in the tumor tissue.

DISCUSSION

The most common treatment for men with metastatic prostate cancer is androgen ablation. With more frequent early use of hormonal ablation, many men will continue this treatment for extended periods of time, often greater than 10 years. The morbidity associated with prolonged hormonal ablation is considerable, including fatigue, osteoporosis, muscle wasting, erectile dysfunction, and decreased libido. To combat these side effects, alternative hormonal strategies have been employed. Intermittent androgen ablation (IAA) was introduced to improve quality-of-life without compromising cancer control. While a large, randomized trial of intermittent versus continuous androgen ablation is ongoing, two intermediate-term reports have shown time to androgen-independence with IAA is equivalent or better(4,14).

Since prostate cancer remains a common cause of death, improvements in the treatment of metastatic prostate cancer are desperately needed. Alternative strategies such as combined androgen blockade, anti-androgen withdrawal, and estrogens have been used to extend the period of disease control. With these needs in mind, we set out to study a novel refinement of IAA by adding finasteride during the 'off-cycle', or androgen-recovery stage.

Following castration, prostate function and growth kinetics are dependent on the relative concentrations of testosterone and DHT. During this phase, DHT is a more potent inducer of prostatic epithelial cellular activity and regrowth(15,16). Using 5- α -reductase inhibitors to limit the amount of DHT reduces the rate of proliferation in the LNCaP human prostate cancer cell line(17). Additionally, numerous growth-inhibiting androgen-response genes have been identified that are preferentially expressed when prostate regrowth occurs in a testosterone-rich,

DHT-poor environment(7). We hypothesized these hormonal conditions may be exploited to limit the growth kinetics of prostate tumors. While the differential effects of testosterone and DHT on normal prostatic regrowth have been established *in vivo*, we sought to extend these studies in a clinically relevant animal model. Since IAA is a common treatment for men with prostate cancer and involves a period of androgen recovery with prostatic regrowth, it seems to be an appropriate and clinically relevant model to test our hypothesis.

Finasteride administered during the 'off-cycle' of intermittent androgen ablation significantly limited prostate cancer tumor growth when compared to two other common treatment strategies, CAA and standard IAA. Mice treated with IAA plus finasteride experienced 75% less tumor growth than the other treatment modalities. This was evident regardless of initial tumor volume.

The ultimate test of any treatment modality is its impact on survival. Mice treated with testosterone plus finasteride during the 'off-cycle' of IAA had a significant survival advantage compared to CAA and standard IAA at all timepoints. At seventy days following treatment, a 3-5 fold survival advantage was evident in the mice treated with IAA plus finasteride.

Additionally, one-time 'off-cycle' continuous hormonal administration, mimicking a prolonged clinical 'off-cycle', resulted in a similar survival advantage for the IAA plus finasteride-treated mice.

Although finasteride can have an anti-proliferative effect on LNCaP cells *in vitro*(18) as well as inhibit androgen receptor-DNA complex formation(19), our results cannot be attributed to

finasteride alone as the mice treated solely with finasteride still had marked tumor growth and behaved similarly to the CAA and standard IAA groups.

LNCaP cells have a mutated AR which can be activated by androgens as well as other steroids such as estrogen(20,21). However, because metastatic androgen-independent human prostate cancers often express similar AR point mutations as those identified in the LNCaP cell line(22), we feel the LNCaP cell line serves as an appropriate animal model. Additionally, since the differential response of our LNCaP tumors to T and DHT mimics that of mouse prostate (wild-type AR) this suggests the mutant AR retained the ability to be androgen-responsive.

The observed tumor volume and survival differences appear to be exclusively due to an altered hormonal environment, as all other measurable variables were consistent between groups. The pellets effectively produced the desired differences in serum and tumor T and DHT. Since estrogen can alter the growth pattern of LNCaP cells(23), we measured estradiol levels in the study groups. No differences in serum or tumor estradiol were observed, therefore the improved outcomes seen in the IAA plus finasteride-treated mice were not due to an estrogen-dependent effect.

Previous work has shown rat prostate, both normal and post-castration, proliferates when exposed to testosterone alone in a DHT-poor environment (after treatment with SK&F 105657, a 5- α -reductase inhibitor) suggesting that testosterone alone is adequate to stimulate some element of the androgenic growth response(24). Similar to our studies in LNCaP cells, they found post-castration exposure to both testosterone and DHT results in markedly increased normal prostate

growth compared to testosterone exposure alone with a 5- α -reductase inhibitor. Our results corroborate these previous findings, extend them to the LNCaP tumor model, and show this growth inhibition to produce a meaningful survival benefit. Similar to Isaacs group (24), we found that following castration, exposure of normal prostate to testosterone plus a 5- α -reductase inhibitor leads to growth but never reaches the size of pre-castration or post-castration plus testosterone prostates (Figure 5).

Compared to humans, male mice have similar serum levels of testosterone but approximately one-third the circulating level of DHT, implying either a decreased level or efficiency of 5- α -reductase activity or increased DHT clearance. We are unaware of any studies measuring 5- α -reductase tissue levels in mice to provide a comparison to human levels. If the concentrations of testosterone and DHT in our studies mimicked human males, in essence if the mice had higher baseline levels of DHT, we suspect the tumor volume and survival differences observed would have been even more dramatic.

Although tumor progression was stunted by the administration of testosterone and finasteride, serum PSA did not differ between the four treatment groups. PSA typically decreases in LNCaP models following androgen ablation since PSA expression is DHT-mediated(12). While we expected tumor volume and serum PSA to be directly related, our discordant results, while surprising are not unprecedented. LNCaP tumors can diminish in size while serum PSA levels simultaneously increase rapidly(18). Further, our LNCaP cell line may have experienced a drift, resulting in either an androgen-independent component or an alteration in PSA production or secretion.

While the bulk of evidence implicates testosterone as growth stimulants for hormonally-responsive tumors, others have also demonstrated testosterone to have growth-suppressant properties. Zhau and colleagues showed that a human-derived metastatic prostate cancer cell line, ARCaP, could be repressed *in vitro* by both testosterone and dihydrotestosterone(25). Tumor growth of this cell line in athymic mice was also suppressed by exogenous androgens. Clinical reports also support these findings. A limited number of men with advanced prostate cancer can experience marked symptomatic improvement when given testosterone(26).

In summary, intermittent androgen ablation with the use of finasteride during the androgen-recovery period ('off-cycle') in a prostate cancer animal model provides the most favorable tumor growth kinetics and improved survival compared to both continuous and standard intermittent androgen ablation. Further genetic and molecular characterization of cell lines treated in this manner will hopefully lead to a more complete understanding of the mechanism. Based on these findings, we are in the process of planning a human clinical trial to study this treatment protocol.

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Figure Legends

Figure 1. Treatment strategy of LNCaP xenograft tumor in nude mice. After the tumor establishment, animals are castrated for 10-14 days, which is considered as the “on-cycle” of androgen ablation during IAA. The animals are then implanted with no pellet, testosterone (T) pellet, finasteride (F) pellet, or both pellets for 14 day or longer. Testosterone implantation mimics intermittent recovery of testicular function during the “off-cycle” of IAA. Controls are continuous androgen ablation (CAA) in the absence or presence of finasteride (F).

Figure 2: a) Mean percent change in LNCaP tumor volume (\pm SEM) during the first ‘off-cycle’ of intermittent androgen ablation ($p=0.0002$), b) percentage of mice with no increase in tumor volume at the end of one cycle of intermittent androgen ablation, c) percent change in tumor volume (\pm SEM) stratified by size of tumor at time of treatment randomization (CAA vs T vs T+F). p -values for $<0.33 \text{ cm}^3$, $0.33\text{-}1.0 \text{ cm}^3$, and $>1.0 \text{ cm}^3$ are 0.038, 0.0022, and 0.027, respectively. * indicates statistically significant

Figure 3: a) Kaplan-Meier survival curve of intermittent androgen ablation with ‘off-cycle’ treatments (CAA-continuous androgen ablation, F-finasteride, T-testosterone, and T+F-testosterone plus finasteride). Euthanasia was performed if tumor diameter $> 2.0 \text{ cm}$, tumor ulceration, or tumor-related morbidity. Log-rank test for trend, $p=0.048$, b) percent survival seventy days following orchiectomy.

Figure 4: a) Kaplan-Meier survival curve of one-time continuous pellet administration following 14 days of castration (CAA-continuous androgen ablation, T-testosterone, and T+F-testosterone plus finasteride), $p=0.034$, b) median survival

Figure 5: a) ventral prostate weights (\pm SEM) following fourteen days of pellet implantation; T versus T+F, $p=0.005$, b) seminal vesicle weights (\pm SEM) following fourteen days of pellet implantation; T versus T+F, $p<0.001$, c) tissue (ng/g) concentrations of testosterone (\pm SEM), $p=0.37$; d) tumor (ng/g) concentrations (\pm SEM) of dihydrotestosterone, $p=0.008$.

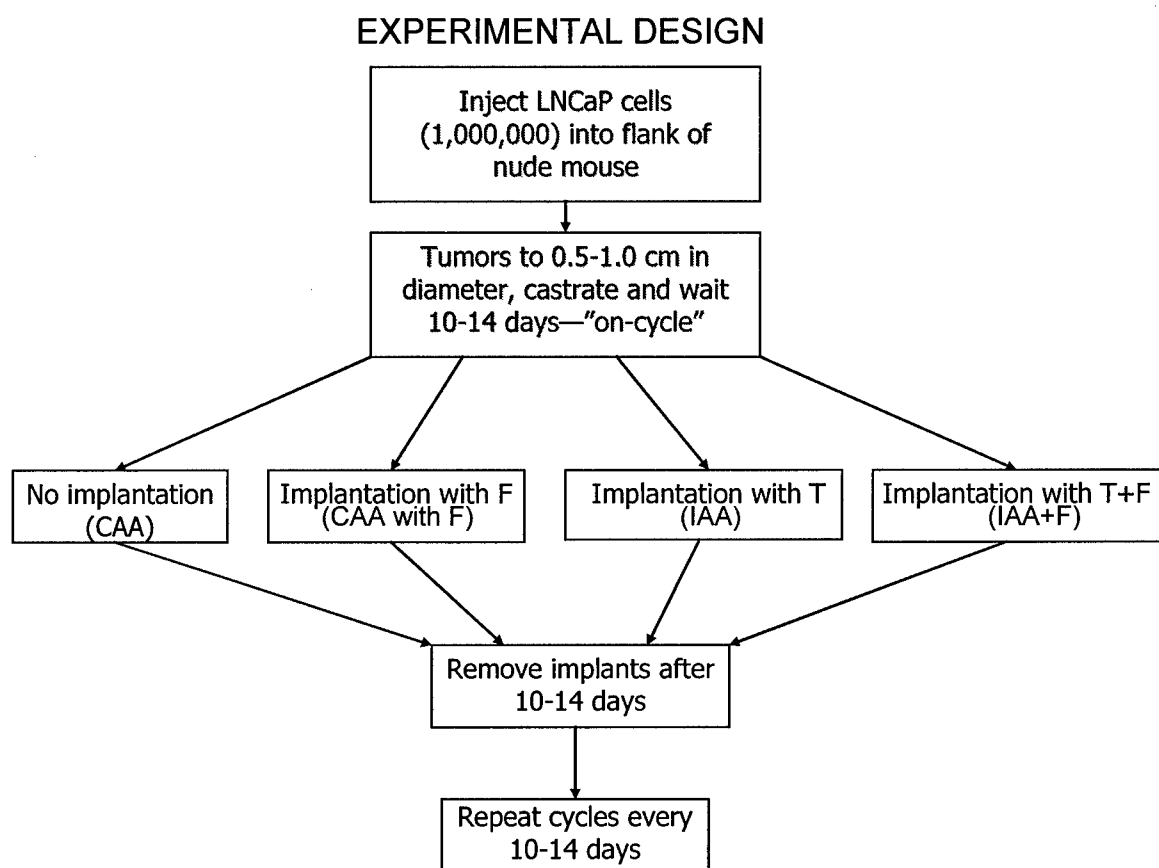


Figure 1

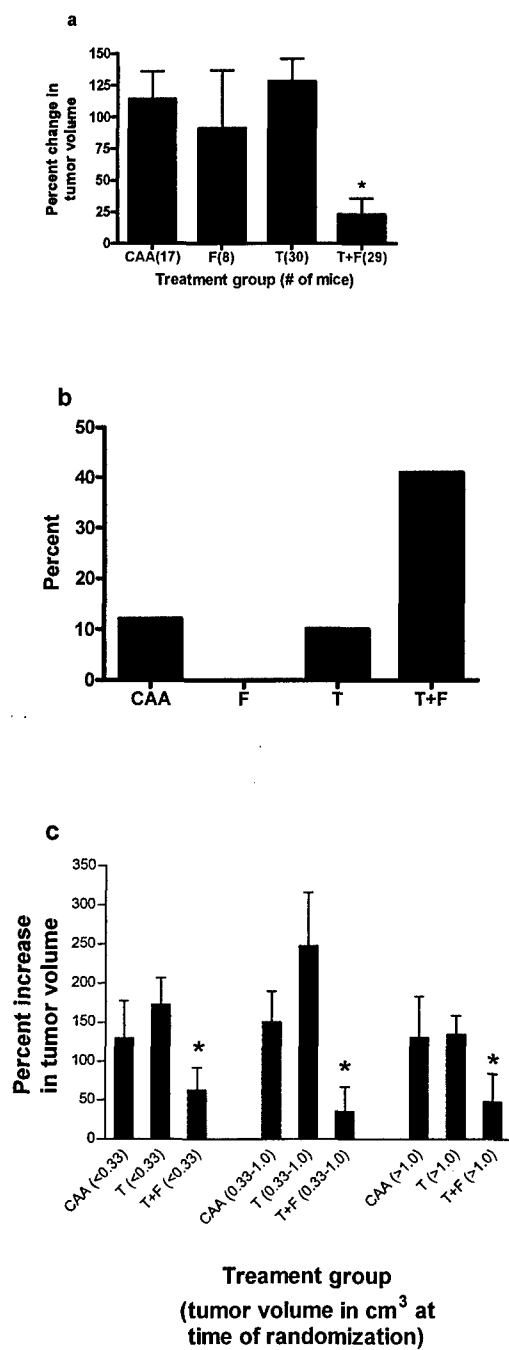


Figure 2.

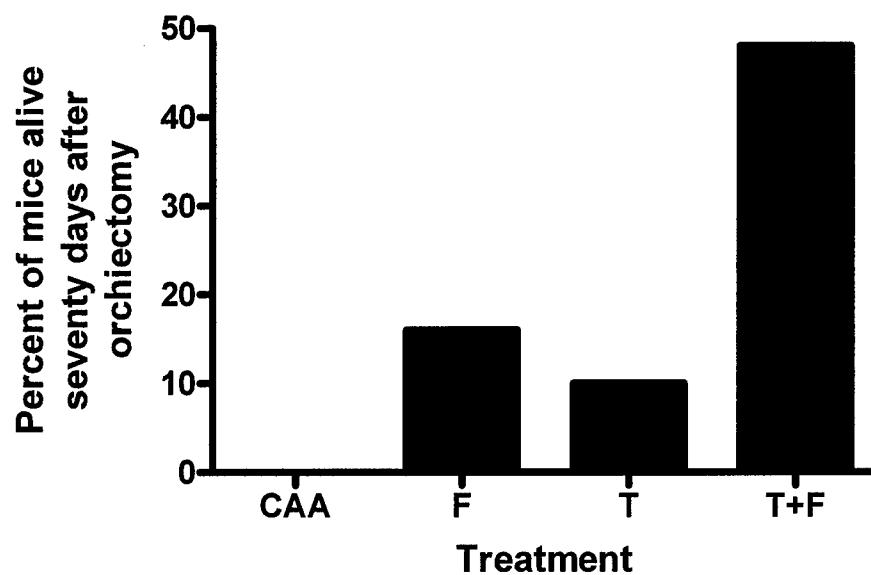
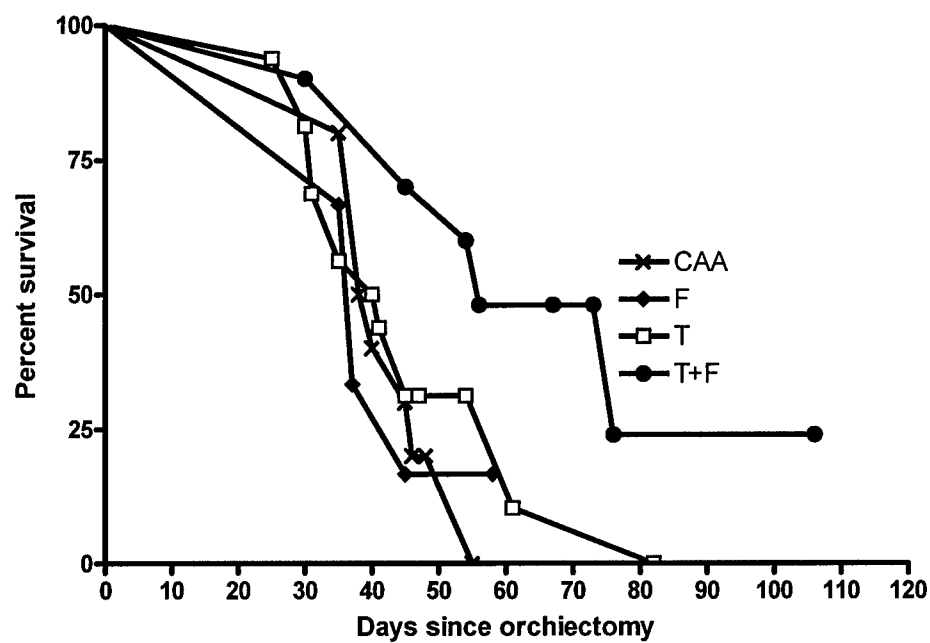


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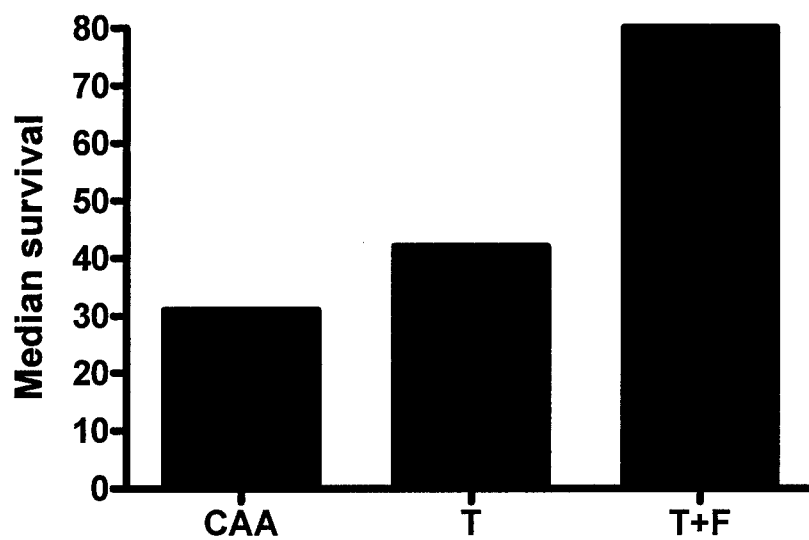
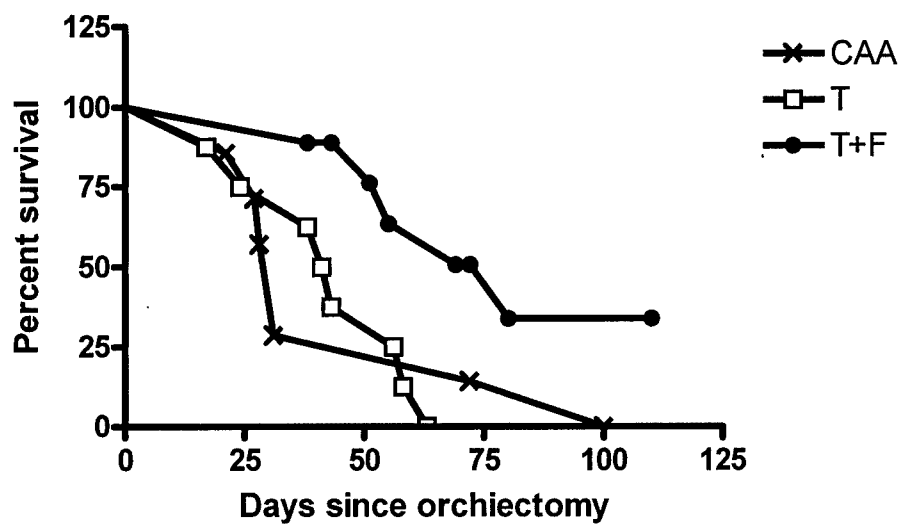


Figure 4.

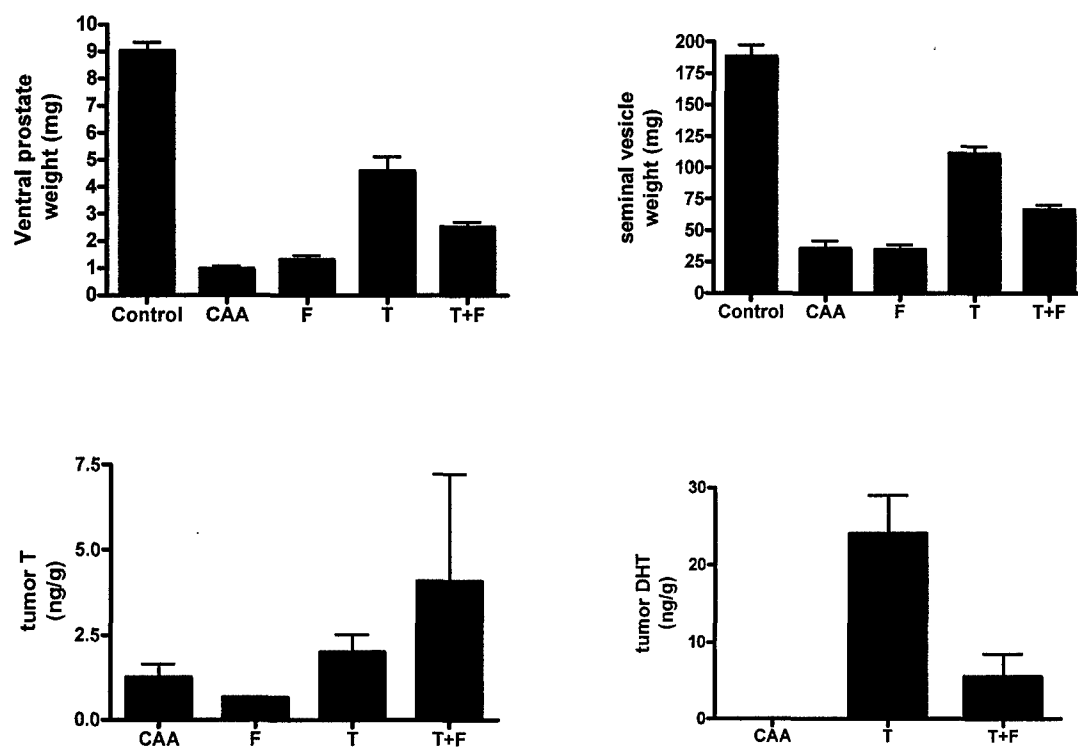


Figure 5.